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(54) Title: REAGENTS AND METHODS FOR DETERMINATION OF ANALYTES

(57) Abstract

Reagents for detecting and enumerating various analytes comprise a specific binding substance for the analyte coupled to a reporter moiety composed of a hydrophobic reporter substance stably associated with a lipid-containing carrier, preferably a liposome. The reagents are particularly useful in cell screening and other diagnostic applications involving labeled specific binding substances, e.g., antibodies.

REAGENTS AND METHODS FOR DETERMINATION OF ANALYTES

Field of the Invention

The present invention relates to reagents which enable reporter substances to become bound specifically to characteristic determinants of various analytes, such as biomolecules, cell-associated structures and other ligands, and to methods for detecting and enumerating such analytes of interest in the presence of different analytes which are not of interest. The invention is of particular utility in cell screening and other diagnostic applications involving labeled specific binding substances, e.g., antibodies.

Description of the Prior Art

Flow cytometry, as the name implies, is concerned with the measurement of cells in flow. Such measurement provides an invaluable research tool, especially in the fields of immunology, hematology and oncology, having important applications in clinical diagnosis and therapeutic decision-making. One of the distinct advantages of flow cytometry in these applications is its ability to produce rapid and accurate analysis of individual cells. Thus, current clinical applications to a large degree are directed to the study of immunologic or neoplastic disorders of peripheral blood, bone marrow, or other tissues that can be disaggregated into single cell suspension. However, flow cytometry is also applicable to the study of subcellular particles and soluble proteins.

Generally, flow cytometry is performed using a single instrument with three integrated components, namely, (i) a sample handling and delivery system, (ii) a detection system and (iii) a digital electronics and data processing system. The sample handling and delivery system serves to direct the sample (cells or particles) in a carefully controlled stream which intersects a narrow beam of light (usually, but not necessarily, laser light) for measurement. The stream containing sample is maintained sufficiently narrow to ensure that single particles or cells pass through the light beam. The detection system may have optical and non-optical components. Non-optical measurement is often accomplished by a volume sensor, which the cells or particles traverse before passing through the light beam for optical measurement. The optical component typically consists of a light source and appropriate lenses to focus the light on the cell or particle to be analyzed. In addition, lenses are provided to collect emitted or reflected light from the cell or particle passing through the light beam. These lenses transmit the light to photodetectors for intensity measurement. When fluorescent light emitted from single cells is measured, sensitive photomultiplier tubes are used. The digital electronics and data analysis system consists of analog to digital conversion electronics and a computer which collects the data and stores it in an appropriate format.

The parameter most often measured in flow cytometry for specific analysis of cells or particles is fluorescence. The combination of intense incident laser light and sensitive photomultiplier tubes makes the flow cytometer well suited for fluorescence

measurements of individual particles. A commonly employed technique in performing fluorescence measurement is to label a cell with a fluorochrome-conjugated antibody or other specific binding substance to selectively identify characteristic cell determinants, such as surface antigens or receptors. Fluorochromes having different absorption and emission characteristics may be combined to allow multicolor analysis of individual cells. Fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE) may be used to practical advantage, in that excitation of such compounds is achievable with smaller, less expensive argon lasers or arc lamps, which do not require water cooling, are relatively simple to maintain and are suitable for immunofluorescence assays. Such compounds are easily covalently conjugated to proteins, such as immunoglobulins.

Flow cytometry has decided advantages as compared with other commonly used cell marker analysis techniques, such as immunofluorescence microscopy, immunocytochemistry and enzyme immunoassay. Specifically, the ability to detect and accurately quantify multiple cellular subpopulations simultaneously is an advantage over bulk methods such as fluorimetry or enzyme immunoassay. Flow cytometry has several notable advantages over manual fluorescence microscopy. First, cells may be scored for positive immunofluorescence thousands of times faster. Second, results are more reproducible and objective and the data obtained may be permanently recorded. Third, the sensitivity of flow cytometry for weakly fluorescing cells is greater than fluorescence microscopy. This feature may be particularly important, for example, in

diagnosing B cell chronic lymphocytic leukemia where the intensity of monoclonal surface Ig is very low. These cells display an average of only 9000 molecules of immunoglobulin on their surface, in contrast to normal B cells, which express an average of 90,000 surface molecules of immunoglobulin per cell.

Flow cytometry also offers the advantage of accurate quantitation of signal intensity, which makes possible quantitation of number of receptors per cell, and the detection of cell cycle phase of individual cells. Furthermore, many flow cytometers may also function as cell sorters, inasmuch as the sample stream may be broken into droplets containing individual particles, which can be electrostatically charged and sorted in an electric field for subsequent morphologic analysis or functional studies. Sorting is mainly used for research applications, but is important on occasion to confirm the identity of a particular cell population of interest in a patient.

In the practice of flow cytometry and immunoassay techniques, it is common to label antibodies directly with fluorescent or radioisotopic reporters. The number of such reporter molecules that may be bound to an antibody molecule without changing its immunoreactivity, however, is quite limited. Moreover, the covalent bonding of a fluorochrome or radioisotopic reporter to an antibody requires compatible reactive groups on each reactant, which imposes another practical limitation on this sort of labeling.

A way of overcoming the above-noted limitation of directly coupling fluorescent reporter to antibodies in fluoroimmunoassays is described in U.S. Patent No. 4,576,912 to S. Yaverbaum et al. The immunoassay

technique disclosed therein involves the use of a reagent comprising a carrier bearing a plurality of closely-packed fluorophores, which is coupled to an immunological reactant competitive with the immunological reactant of interest. The fluorophores are sufficiently closely packed as to exhibit self-quenching and the reagent is capable of undergoing chemical treatment to release the fluorophores. A test sample and the reagent are mixed with a solid phase bearing a specific binding substance for the immunological reactant of interest, with which the reagent competes. After the competitive-binding reaction occurs, the bound immunological reactants and unbound immunological reactants are separated. The carrier, in either or both separated portions, is then chemically treated, or lysed, to liberate the otherwise quenched, closely-packed fluorophores to greatly enhance the measured fluorescence. The fluorescence intensity of the liberated fluorophores is then compared to a standard of known concentration to determine the amount of immunological reactant in the sample. This immunoassay technique is not without certain drawbacks, however, in that it utilizes covalent coupling of the reporter molecules to the carrier and a subsequent chemical reaction, which is typically a time consuming enzymatic reaction, to effect reporter release prior to detection.

In order to avoid direct labelling of fluorescent reporters to antibodies for use in flow cytometry and fluorescence microscopy, it has been proposed to use reagents comprising antibodies attached to liposomes loaded with dye molecules entrapped in the aqueous phase inside the liposomes. The increased number of

reporter molecules per antibody has been found to enhance signal amplification. Furthermore, encapsulation of the reporter within the liposome permits a wider range of reporter molecules to be used, including those that cannot be directly bound to antibody. See: A. Truneh et al., J.Immunol. Methods, 100:59-71 (1987) and references cited therein; and U.S. Patent No. 4,372,745 to R. Mandle et al.

Similar reagents have been proposed for use in other diagnostic applications. The patent literature discloses immunoassays utilizing reagents in which a specific binding substance for an immunoreactive substance of interest is fixed on lipid membrane-containing microcapsules, having a hydrophilic marker encapsulated in the microcapsules. In performing such immunoassays, a test sample containing the immunoreactive substance of interest is mixed with the reagent and a source of complement causing the marker to be released from the microcapsules, after which its presence or quantity is determined by appropriate analysis. See for example, Japanese Patents 60159652-A and 60017359-A. According to the specific embodiments disclosed in these references, the microcapsules are liposomes which are fixed to the specific binding substance via covalent bonds. See also: Japanese Patents 60138464-A, 60138465-A and 60138466-A. An additional advantage of this method is the ability to measure bound reagent in the presence of unbound reagent. However, the method also has several disadvantages. First, complement is a labile reagent and false negatives may result in this method from failure of complement to properly lyse liposomes and release reporter. Second, if the disclosed reagent

were used to determine cell surface associated structures, complement could cause lysis of cells as well as liposomes, releasing substances which may interfere with measurement of some reporter molecules. Third, microenvironments within some test samples (e.g., low pH) may cause significant leakage and nonspecific release of some reporter molecules (e.g., carboxyfluorescein; P. Machy et al., Proc. Nat. Acad. Sci. USA, 79:4148 (1982)).

Time-resolved immunofluorometric assays have been disclosed in which rare earth metals are used as reporters. See Alfthan et al., Am. Biotech. Lab., 6, (6), 8-13 (1988) and references cited therein. Such assays generally involve use of aminopolycarboxylic acid chelates of rare earth metals (in practice Eu^{3+} or Tb^{3+} complexes) to label antibodies. Aminopolycarboxylic acid chelates bind the metal ion strongly enough to keep it associated with antibody during the antigen-antibody reaction, but have minimal fluorescence. Rare earth fluorescence is developed by extracting it into a mixture of an aromatic diketone, which replaces the aminopolycarboxylic acid as chelator, a detergent to solubilize the highly insoluble diketone through micelle formation, and a Lewis base to make the fluorescence yield less sensitive to solvent quenching. In order for the extraction to take place in a reasonable time, acid pH must be used. The fluorescence of the resulting chelates is characterized by relatively long lifetimes compared with those of typical sources of fluorescent "noise", e.g., serum components. If a pulse of light is used to excite sample fluorescence, the "noise" fluorescence decays much more rapidly than the

antibody-related fluorescence. This makes it possible to wait until "noise" fluorescence becomes minimal before detecting antibody-related fluorescence (hence the name time-resolved fluorescence), thereby improving signal:noise ratio. However, measurement time is increased.

A tissue or cell specific drug-delivery reagent comprising a polysaccharide-coated liposome is described in Sunamoto et al., Biochem. Biophys. Acta, 898:323 (1987). The polysaccharide moiety (pullulan) is covalently conjugated to cholesterol and to antibody IgM fragment. One method described for determining attachment of the liposomes to the surface of target cells was incorporation of a hydrophobic fluorescent probe, terbium trisacetylacetonate, in the lipid membranes and examination of the stained cell surface by fluorescence microscopy.

An enzyme-linked immunosorbent assay (ELISA) has been proposed for quantitation of human T cells and their subpopulations. J. Endl et al., J.Immunol. Methods, 102:77-83 (1987). Because this assay measures absorbance, it is less sensitive than immunofluorescence assays. The reporter in this system utilizes an enzyme, β-galactosidase, which is also present in blood monocytes and neutrophils. Therefore, this technique is not well-suited to determinations of cell subtypes in whole blood, and often, a prior separation must be performed to remove interfering cells or cellular material capable of causing false positives.

A need exists for reagents and methods for detection and quantitative determination of analyte, particularly in the area of cell analysis, wherein assay sensitivity is enhanced, reagent stability under

different reaction conditions is maintained, and the requirement for expensive equipment and highly skilled personnel is obviated.

SUMMARY OF THE INVENTION

The present invention provides a novel approach to detecting and enumerating various analytes by use of a reagent comprising a specific binding substance for the analyte, which is coupled to a reporter moiety composed of a lipid-containing carrier bearing a reporter substance. The reagents of the invention are characterized by having hydrophobic reporter substance stably associated with the lipid component of the carrier. As compared with the aforementioned prior art reagents, the reagents of the invention afford greater flexibility regarding the amount and type of marker substance that may be incorporated therein. Moreover, unlike some of the prior art reagents, the reagents of the invention do not require covalent coupling of reporter substances to a specific binding substance or to a carrier. Furthermore, because the reporter substance is stably associated with the lipid component of the carrier, there is no appreciable leakage of the reporter substance from the reagent under conditions in which the integrity of the lipid component is maintained.

The reporter substance is readily extractable from the reagent, which permits the use of relatively simple and inexpensive analytical devices and techniques, e.g., fluorometry. The reagents of the invention may, if desired, be used in conjunction with a hydrophilic reporter substance encapsulated within

another reagent, which allows differential extraction of hydrophobic and hydrophilic reporter substances, thereby increasing the number of simultaneous determinations that may be made by relying on solubility, as well as other detectable differences, e.g., spectral differences, to distinguish among analytes.

In one embodiment, the reagent includes fluorescent compounds, as the reporter substances, bound to liposome carriers. Such reagents provide increased fluorescence efficiency due to the local non-aqueous environment. Fluorescence efficiency may be increased even further by extraction and concentration of the fluorescent reporter prior to fluorescence measurement.

The present invention also provides a method for producing the aforesaid reagents by preparing liposomes incorporating a hydrophobic reporter substance in stable association with the lipid component of the liposomes, which are then contacted with the binding substance under conditions causing the liposomes to couple to the binding substance. In an exemplary preparative method, sulfhydryl groups are incorporated in the liposomes and the binding substance, which react to form a disulfide bond between the liposomes and the binding substance.

The present invention further provides several methods for the efficient determination and quantitation of various analytes of interest in test samples or specimens. In accordance with one method of the invention, immunoreactive ligands expressed on the surface of cells are determined or quantitated using the reagents of the invention. Similarly, a subset

cell type in a cell population may be determined or quantitated using the reagents of the invention. Another method of the invention enables determination of the proportion of a subset cell type relative to a cell population which consists of a defined number of subset cell types, each subset cell type having a characteristic surface ligand. These methods are described in detail hereinbelow.

The methods of the invention may be used in conjunction with, and in certain instances as a replacement for many of the current applications of flow cytometry in clinical laboratories where the goal is to screen for changes in cell frequency. Information obtainable by extraction in accordance with the present invention on the one hand, and by flow cytometry, on the other hand, is not identical. Extraction data provides information concerning changes in cell number and antigen density while flow cytometric data distinguishes between these two parameters. However, for many applications it would be cost efficient to single out the samples in which one or both parameters are significantly altered, and, if necessary, to distinguish between parameters, to reanalyze those samples by flow cytometry. In many cases, the same reagent could be used for extraction and for flow cytometric reanalysis.

Other advantages of the present invention will be apparent to those skilled in the art upon consideration of the drawing and detailed description presented herewith.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph representing the correlation between the number of Lyt-1 bearing cells and fluorescence intensity of the reporter substance extracted from a specific antibody-carrier- reporter conjugate (anti-Lyt-1-liposome-$DiOC_{18}[3]$) bound selectively to Lyt-1 bearing murine spleen cells.

Fig. 2 is a graph representing correlation of varying percentages of $H\text{-}2K^k$ bearing cells in a mixture of $H\text{-}2K^k$ and $H\text{-}2K^d$ bearing cells with fluorescence intensity of a reporter substance extracted from a specific antibody-carrier-reporter conjugate (anti-$H\text{-}2K^k$-liposome-S467) selectively bound to $H\text{-}2K^k$ positive cells in the cell mixture. The $H\text{-}2K^k$ cells were analyzed in a mixture prepared by admixing increasing numbers of B10.A ($H\text{-}2K^k$) murine spleen cells with Balb/c ($H\text{-}2K^d$) murine spleen cells.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, various analytes are qualitatively or quantitatively determinable by use of a reagent comprising at least one specific binding substance, capable of selective interaction with a characteristic determinant of the analyte, and a reporter moiety coupled to the binding substance. The reporter moiety of the reagent is characterized in that it comprises a detectable reporter substance and a carrier having a lipid component, with the reporter substance being stably associated with the lipid component of the carrier.

The term "couple" or "coupled", as used herein, refers to the stable association between two substances

brought about by any chemical, physical, or physico-chemical binding, such as by covalent bonds, hydrogen bonds, polar attraction, van der Waals' attraction, or adsorption, including also biospecific adsorption.

The expression "stably associated with", used herein to describe the manner in which the reporter substance is joined to the lipid component of the carrier, is intended to mean that the reporter substance remains associated with the lipid component of the carrier even when exposed to conditions or agents which tend to cause leakage or loss of materials present in any aqueous phase also associated with the carrier, such as decreased pH causing neutralization and leakage of anions of weak acids, see e.g., P. Machy et al., Proc. Nat. Acad. Sci., U.S.A., 79:4148 (1982); complement-induced release of reporter upon binding of complement to carrier-associated antigen-antibody complexes, see e.g., Monroe, Amer. Biot. Lab, 5:10-19 (1987); and the like. Such stable association between the reporter substance and the lipid component of the carrier is lost only upon manipulations which disrupt or destroy the integrity of the lipid phase of the carrier, for example, by contact with detergents or lipid solvents which dissolve or disperse the lipid phase.

On a quantitative basis, the expression "stably associated with" signifies that greater than 95% of the reporter substance initially associated with the lipid component of the carrier upon preparation of the reagent remains associated therewith for at least a week (as detected by physical separation of carrier from dissociated reporter moiety). Minimum acceptable

stability of assocition for a given application of the reagent will, of course, be determined by the length of time between preparation of the particular reagent desired and its intended use.

The term "analyte", as used herein, refers to any constituent of a test sample or specimen whose presence or quantity may be determined by selective interaction with a specific binding substance. "Analyte" thus encompasses a large number of substances of biological or medical interest. The analyte of interest may be associated with a cell, for example, a component of the cell membrane, cytoplasm or nucleus. Such cell associated structures may include, for example, membrane proteins or glycoproteins, cell surface antigens of either host cell or viral origin, histocompatibility antigens, or membrane receptors. The test sample may thus comprise a cell suspension in a suitable biological or synthetic medium. The analyte of interest may also be present in test samples or specimens of varying origin, including especially biological fluids such as serum, plasma, urine, cerebrospinal fluid and amniotic fluid. In certain instances, the analyte may be absorbed or immunologically captured from biological fluid, e.g., serum, onto a suitable solid support, e.g., polystyrene spheres.

A subclass of analytes which may be beneficially determined by the practice of this invention are ligands whose presence or quantity may be determined due to binding affinity for an immunospecifically recognizable complementary binding substance, the two substances together forming an antigen-antibody complex, immune complexes or aggregates which may be determined by the methods of the invention.

Representative of substances included by the term analyte, as used herein, are antigens, antibodies, haptens and various other substances having a naturally occurring receptor. The complementary binding substance for determination of an antigen is an immunospecific antibody which recognizes the antigen of interest. Conversely, if antibody is the analyte to be determined, the eliciting antigen may be used as the complementary binding substance. The term "antibody", as used herein, includes serum proteins, generally known as immunoglobulins, as well as monoclonal antibodies, and antibody fragments. Likewise, other analytes and their complementary binding substances (and vice versa) include receptors-hormones and avidin-biotin. Examples of other analytes and complementary binding substances are DNA or RNA oligomers and their complementary nucleotide sequences, peptides and antisense peptides (i.e., peptide made from an RNA sequence complementary to that encoding a peptide analyte), the Fc receptor portion of mouse IgG and protein A, and sugar or carbohydrate sequences and complementary lectin (e.g., mannose and concanavalin A). Other analyte-complementary binding substance pairs that may be determined using the methods of the invention will be apparent to those skilled in the art.

The term "determinant" is used herein in its broad sense to denote an element that identifies or determines the nature of something. When used in reference to an analyte, "determinant" means that portion of an analyte involved in and responsible for selective binding of the specific binding substance, the presence of which is required for binding to occur.

The expression "reporter substance" is used herein to refer to the molecular structure associated with the reagent of the invention whose direct detection or measurement by physical or chemical means is indicative of the presence of the analyte or ligand of interest. Examples of useful reporter substances include, but are not limited to the following: molecules or ions detectable by their absorbance, fluorescence, phosphorescence, or luminescence properties; molecules or ions detectable by their radioactive properties; and molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. Fluorescent compounds are particularly suitable for use as reporter substances in the practice of this invention.

Among the cell types which may be determined according to the invention include any cells expressing a characteristic integral membrane determinant which is capable of selectively binding with another molecule, which includes cells of human or animal origin, cultured cells, cell fractions, as well as some single-celled organisms or virions. Cells of particular interest in current technology include human and animal hemopoietic cells, including stem cells, progenitor cells for different lineages, cells at different stages of differentiation within a lineage, and mature forms of various lineages. Of particular interest in diagnostic, therapeutic and research applications are mammalian lymphocytes, including B cells, T cells, and recognized T cell subgroups, comprising helper T cells, suppressor T cells and cytotoxic T cells.

The reagents of the invention comprise three principal components: a carrier, at least one specific

binding substance, and a reporter substance. The specific binding substance is responsible for selective interaction between the reagent and the analyte of interest. The specific binding substance employed in the reagent must exhibit selective recognition for the characteristic determinant of the analyte. Based on such selective recognition, the specific binding substance is capable of selective interaction and binding with the analyte in a test medium to form a substance which is physically or chemically separable from the medium and from unreacted reagent. The specific binding substance is coupled to the carrier component of the reagent. The carrier has a lipid component and preferably comprises a biological or nonbiological lipid membrane. Examples of suitable biological membranes include red blood cells or red blood cell ghosts produced by hemolysis. Suitable nonbiological membranes are provided, for example, by liposomes.

Liposomes are unilamellar or multilamellar lipid vesicles which enclose a three-dimensional space. Various methods for preparing liposomes are known to those skilled in the art, as are techniques for encapsulation of hydrophilic substances therein.

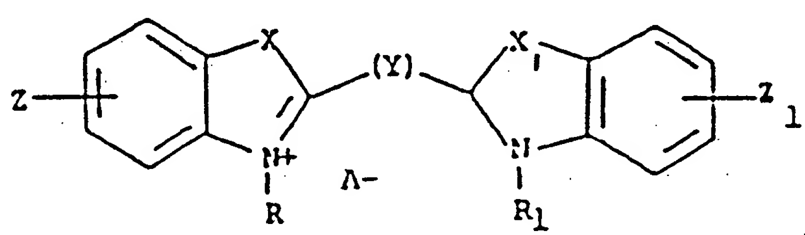
The reporter component of the reagent comprises a substance having a detectable or measurable physical property. The determination of reporter substance provides a basis for detecting the interaction of the reagent with the analyte of interest. The extent to which the reagent interacts with the analyte of interest allows detection or quantitation of the analyte. In the reagents of the invention, the reporter substance is stably associated with the lipid

component of the reagent carrier. When the carrier is a liposome, the reporter substance is selected on the basis of its capacity to become embedded in the lipid bilayer during preparation of a liposome suspension.

The reporter substance may comprise a substance which is entirely hydrophobic in character, or may comprise a moiety having at least one hydrophobic portion or domain serving to anchor the reporter molecule to a membrane bilayer or other lipid component. In the latter instance, the hydrophobic portion of the reporter molecule should associate the molecule to lipid sufficiently to cause it to remain stably associated with the lipid, even in an agitated solution or disrupted cell suspension.

Generally, the reporter substance may comprise any molecule or ion which is capable of becoming stably associated with lipid and is detectable based on light absorbance, fluorescence, luminescense, phosphorescence, radioactivity, or nuclear magnetic resonance or paramagnetic properties. A suitable class of reporter substances comprises fluorochrome compounds selected from the group of cyanine, acridine, pyridine, anthraquinone, coumarin, quinoline, xanthene, phenoxazine, phenothiazine and hexatriene dyes and derivatives thereof.

A particularly suitable class of reporter substance is represented by the formula:



wherein R and R_1 are the same or different and

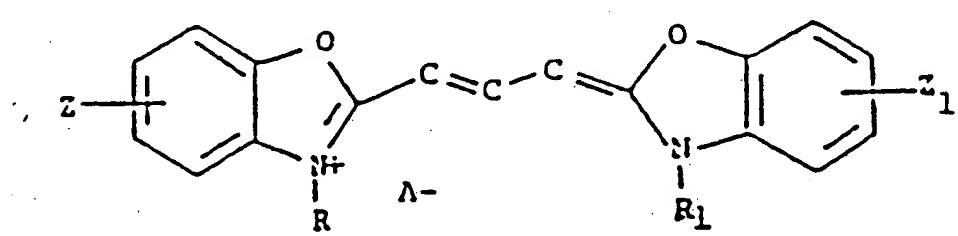
represent substituents independently selected from the group of hydrogen, alkyl, alkenyl, alkynyl, alkaryl or aralkyl, the hydrocarbon chains of which having from 1 to 30 carbon atoms, and being linear or branched, said substituents being unsubstituted or substituted with one or more non-polar functional groups, one of R or R_1 having at least 12 linear carbon atoms, and the sum of the linear carbon atoms in R and R_1 being at least 23;

X and X_1 may be the same or different and represent O, S, $C(CH_3)_2$ or Se;

Y represents a linking group selected from -CH=, -CH=CH-CH=, -CH=CH-CH=CH-CH=, or -CH=CH-CH=CH-CH=CH-CH=;

Z and Z_1 may be the same or different and represent substituents selected from the group H, alkyl, OH, NH_2, COOH, $CONH_2$, SO_3H, SO_2NH_2, $NHNH_2$, NCS, NCO, CONH-alkyl, CON-$(alkyl)_2$, NH-acyl, O-alkyl, NH-alkyl, or $N(alkyl)_2$, SH, S-alkyl, NO_2 or halogen, the alkyl groups comprising said Z substituents having from 1 to 3 carbon atoms; and A represents a biologically compatible anion.

A second particularly preferred class of reporter substance is represented by the formula:



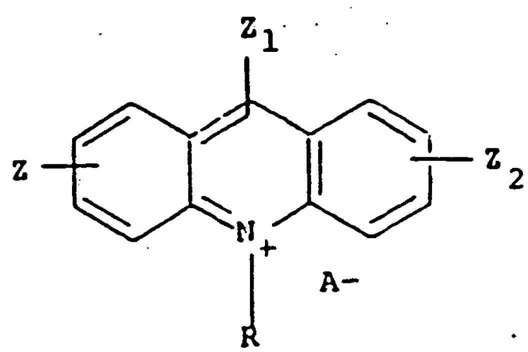
wherein R and R_1 are the same or different and represent alkyl substituents, having from 1 to 30 carbon atoms, and being linear or branched, unsubstituted or substituted with halogen, one of R or

R_1 having at least 12 linear carbon atoms and the sum of the linear atoms in R and R_1 being at least 23;

Z and Z_1 may be the same or different and represent substituents selected from the group H, or lower alkyl having from 1 to 3 carbon atoms; and A represents a biologically compatible anion.

Of the last preceding class of compounds, preferred compounds include 3,3'-di-n-octadecyloxacarbocyanine perchlorate, 3-n-pentyl-3'-n-octadecyloxacarbocyanine iodide, 3-n-octyl-3'-n-octadecyloxacarbocyanine iodide, 3-n-propyl-3'-n-eicosanyloxacarbocyanine iodide, and 3-n-propyl-3'-n-docosanyloxacarbocyanine iodide.

A further particularly preferred class of reporter substances is represented by the formula:



wherein R represents a substituent selected from the group of alkyl, alkenyl, alkynyl, alkaryl or aralkyl, the hydrocarbon chain of which is linear or branched, said substituent being unsubstituted or substituted with one or more non-polar functional groups, and having at least 23 linear carbon atoms;

Z, Z_1 and Z_2 may be the same of different and represent substituents selected from the group H, alkyl, OH, NH_2, COOH, $CONH_2$, SO_3H, SO_2NH_2,

$NHNH_2$, NCS, NCO, CONH-alkyl, CON-$(alkyl)_2$, NH-acyl, O-alkyl, NH-alkyl, or $N(alkyl)_2$, SH, S-alkyl, NO_2, halogen, the alkyl groups comprising said Z substituents having from 1 to 3 carbon atoms; and A represents a biologically compatible anion.

Of the preceding class of compounds, a suitable compound is 3,6-bis(dimethyl- amino)-10- n-hexacosanyl acridinium iodide. Other suitable fluorochrome compounds are 4-[4-didecylaminostyryl]-N-methyl-lpyridinium iodide, and N-3[3-Sulfopropyl]-4-[p-didecylaminostyryl] pyridinium inner salt.

As used in the preceding description of the reporter substance, the term "non-polar functional group" refers to substituents such as O-alkyl, S-alkyl, halogen, $N(alkyl)_2$, Se-alkyl, NO, CN, CO-alkyl, C=N-alkyl, $-SiMe_3$, O-$SiMe_3$, and the like.

Alternatively, the reporter substance may comprise a substantially hydrophobic chelate-metal complex, preferably wherein the chelate-metal complex comprises a metal ion selected from the transition metal series whose atomic number is from 21-29, the lanthanide series whose atomic number is 59-66 and the actinide series whose atomic number is 91, said complex being detectable by nuclear magnetic resonance or luminescence. The chelate-metal complex may also comprise a paramagnetic metal ion selected from the group of Gd, Cr, Dy, Ni, Cu, Fe and Co.

The reporter substance may comprise a substantially hydrophobic substance incorporating a detectable radioisotope. The radioisotope incorporated into the reporter substance is preferably selected from the group of radioactive hydrogen, carbon, nitrogen, phosphorus, fluorine, chlorine, iodine, sulphur and

selenium.

The foregoing classes of hydrophobic reporter substances, which are suitable for incorporation in the reagents of this invention, are described in further detail, along with preparative procedures for specific compounds, in copending U.S. Patent Application Serial No. 189,192, which is commonly assigned with the present application. The entire disclosure of Serial No. 189,192 is incorporated in this application by reference, as if set forth herein in full. These reporter substances are characterized by having relatively long chain hydrocarbon substituents or "tails" that impart the requisite hydropholicity/ lipophilicity for stable association with the lipid component of the carrier. Once these compounds become bound to lipid, they cannot easily dissociate. Consequently, the reporter substance does not leak from the reagent and is not liable to be transferred to biomolecules or cell-associated structures.

In preparing the reagents of the invention, the reporter substance is caused to be stably associated with the lipid component of the reagent carrier. The reporter substance may be included during conventional liposome preparation, which results in the reporter becoming stably associated with membrane in the final liposomes. Alternatively, the reporter substance may become associated with the lipid component of the carrier, for example, by incubation of the reporter substance with preformed liposomes. In certain applications where liposomes serve as the carrier, it may be desirable to encapsulate additional reporter substances within the liposome vesicles.

Having produced the carrier component containing

the reporter substance, the reporter-containing carrier may be contacted with a specific binding substance under a variety of conditions causing coupling of the specific binding substance to the carrier. A number of suitable techniques are available for carrying out this coupling reaction. For example, an activatable group may be affixed to a carrier component either during or after its preparation. Attachment of a chemically activatable group to the carrier component may be covalent or noncovalent. The activatable group is capable of reacting with an additional activatable group on the specific binding substance. Addition of an activating agent allows reaction of the activated group on the carrier component with the activated group on the specific binding substance and chemically couples the binding substance to the carrier to form the reagent.

To achieve covalent coupling, the activatable group associated with the carrier and the activatable group associated with the specific binding substance should be highly reactive species such as amino, hydrazino, aldehyde, isothiocyanate, carboxyl, or sulfhydryl groups. Suitable covalent linkages between the carrier component and the specific binding substance include disulfide, ester, amide, triazine, acetal, imine, hydrazone, semicarbazone, urea, thiourea, ether or thioether linkages, or a linkage formed by a reduced Schiff base. One method of effecting chemical coupling involves providing a sulfhydryl functionality as each of the activatable groups, which react to form a disulfide bond between the carrier component and the specific binding substance. Such a method is described in Leserman et

al., Nature, 228:602-04 (1980).

As an alternative preparation of reagent, the activatable group on the specific binding substance may comprise a domain or a reactive site on the specific binding substance molecule. For example, the specific binding substance may comprise antibody and the specific binding domain may comprise the antibody Fab domain. The Fc domain of the same antibody molecule may be coupled directly to the activatable group associated with the carrier without hindering reactivity of the Fab domain. See, for example, European Patent Application No. 0088695 by Cytogen Corp. (Application No. 83400461.6; Publication date: September 14, 1983). Alternatively, the Fc domain of the same antibody molecule may be coupled directly to a lipophilic compound which associates with the lipid component of the carrier.

The methods of the invention involve use of the reagent to measure analyte quantitatively or qualitatively. One method generally involves reacting the reagents with analyte in a fluid sample to form a complex. The complex is physically or chemically separated from unreacted sample constituents and from unreacted reagent. Measurement of the reporter substance in the complex or in the unreacted reagent provides information enabling determination of the presence or quantity of analyte in the sample. A typical method generally involves reaction of the reagent with a cell suspension containing cells with which the reagent is capable of forming complexes. The complex formed between the reagent and the cells of interest within the suspension is separable from the suspension and the presence or amount of reporter

substance in the complex or in the unreacted reagent may be determined.

Specific techniques for the quantitative and qualitative determination of analyte include immunoassay of the analyte in a solution, for example, the determination of a protein component or a drug in a sample of plasma, serum, or urine. Prior to performing the measurement, it may be advantageous in certain instances to first immobilize the analyte of interest from the sample on a substrate, such as polystyrene microspheres or other known solid supports. The reagent is incubated with the sample to form complexes or aggregates between the reagent and the analyte of interest. The complexes which are formed may be physically separated by centrifugation or washing from the other components in the reaction medium. The reporter substance is then extracted from the complexes or from the unbound reagent and measured to provide an indication of the analyte content of the sample.

The reporter substance present in cell-associated complexes may be determined in several ways. The measuring techniques involve first separating cells and associated cell complexes from the reaction mixture. The presence of reporter substance complexed with the cells of interest may be determined directly from measurement of the individual cells in solution using automated cytometric methodology. Alternatively, the reporter substance may be separated by extraction from the other components of the cell complexes. Extraction of the reporter substance may be desireable to avoid potential interference with the measurement due to cellular material.

The reagent of the invention has utility in

determination of specific cell types or subtypes within a cell population including determination of total numbers of the cells of interest within a sample cell suspension, or determination of the proportion of a cell subtype within a cell population. Cell subtypes within a population may include cells of differing lineage or cells of the same lineage at different stages of differentiation. Different lineages of cells are characterized by expression of characteristic antigens or ligands. For example, B cells from mammalian blood samples express surface ligands distinct from those expressed by T cells from the same sample. Quantitation of one type of cell from the sample may be important in assessing certain pathological conditions. For example, an abnormally large proportion of a single B cell clone in a patient's blood may be indicative of a leukemic condition. Cells from the same lineage at different stages of differentation are also distinguishable by expression of characteristic antigens or ligands. For example, as a B lymphocyte develops from a stem cell to a pre-B cell and ultimately to a mature B cell, the cell membrane markers change in a predictable manner as the cell matures. Whereas a mature B cell expresses immunoglobulins as ligands on the cell membrane, a pre-B cell expresses only cytoplasmic immunoglobulin heavy chains, which provides the basis for differential reactivity of these cell types with reagent and their subsequent determination. Differential expression of ligand can further provide a basis for assessing pathogenesis such as viral infection. Virally infected cells may express viral markers which are absent from uninfected cells within the cell population. These

viral markers may be found intracellularly or on the cell membrane.

In detecting and enumerating cells of interest within cell populations or cell suspensions in accordance with the invention, the reagent is coupled to a specific binding substance having selective affinity for the characteristic marker of the cell type or subtype of interest. Upon incubation of the reagent with a test sample, selective binding of the reagent occurs with the cell type of interest to form a complex between the targeted cell type and the reagent. The quantity of reactant to be incubated with a given sample volume, the time of incubation, and other reaction conditions such as temperature may vary with the nature of the cells of interest. The reaction conditions must be such as to permit selective reaction between the cell type of interest and the reagent to form complexes. The resulting complexes are separated from the uncomplexed reagent within the the reaction medium. Detection of the reporter substance present within the separated complexes indicates the presence of the cell type of interest or target cells within the population. Quantitation of the reporter substance provides information as to the number of target cells within the population and the average antigen or ligand density per target cell.

In a further embodiment of the method of the invention, identification or quantitation of more than one target cell type or subtype within the same population can be achieved. Such a cell population may contain or be suspected of containing two subset cell types, among other subsets, which, for purposes of this discussion, are designated subtype A and subtype

B, and which express ligand X and ligand Y, respectively, as characteristic markers. The determination of the presence and/or quantity of cells of cell subtype A and cell subtype B in the sample in clinical or experimental analysis can be efficiently done with the reagents of the invention following a number of related protocols. These methods involve preparing one reagent which selectively reacts with cell subtype A via a selective binding reaction and complex formation between ligand and the one reagent. Specific binding substance for the reagent is chosen such that it selectively reacts with ligand X. Another reagent is prepared which includes a specific binding substance which selectively interacts with ligand Y expressed by cell subtype B.

In one protocol, discrete samples of the cell population of equivalent volume and cell concentration may be reacted with the one reagent and the other reagent, respectively. Following reaction and separation of complex from each reaction, the reporter substance may be quantitated from each sample to provide an indication of the amount of A and the amount of B in the population. In this embodiment, the reporter substance used in the one reagent and in the other reagent may be the same or different. Of course, additional reagents may be prepared and used as needed for determination of other different cell subtypes.

In an alternative protocol, the one reagent and the other reagent may be reacted in the same sample of the population by adding and incubating both reagents to form discrete complexes within the sample. The resulting complexes are discrete due to the selective affinity of each reagent for its respective different

cell subtypes. When the complexes are separated from the reaction, the total complexes will contain cell complexes formed by both selective reactions. In this embodiment, the reporter substance in the one reagent must be distinguishable or separable from the reporter substance in the other reagent to permit distinct readings of the two cell types by determination of the two reporter substances. In this regard, the reporters may comprise substances having distinct spectral properties, by which is meant sufficiently differing excitation wavelength(s), emission wavelength(s), or fluorescence lifetime(s), such that detection of the presence of one reporter substance is not compromised by the presence of any other reporter substance, i.e., the values determined for one are not significantly affected by the presence of another. The use of such reporter substances permits individual measurement of each reporter independently of one or more other reporters present in the complexes. Alternatively, the one reporter may be physically separable from the other(s), for example, by reason of its solubility.

In another embodiment of the method of the invention, the second reporter is a substantially hydrophilic substance and is encapsulated within, rather than associated with, the lipid component of the second reagent. Methods of preparing liposomes containing encapsulated substances in the aqueous phase are well known in the art. Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980). Upon separation of the two types of complexes from the reaction sample, the complexes containing multiple cell subtypes may be physically disrupted and separated into distinct aqueous and lipid phases. The lipid phase will contain

the first reporter substance associated therewith and the aqueous phase will contain the second reporter substance associated therewith, and the phases may be separated to permit independent measurement of the reporter substance in each phase.

A further embodiment of the method involves determining the proportional occurrence of a subset cell type within a cell population. A population of cells may comprise numerous defined subset cell types, for example, a population of T lymphocytes may comprise a number of specialized subgroups, each equipped for a particular T cell function and each expressing characteristic surface antigens. In order to determine the proportional occurrence of a subset type, it is necessary to resolve both total cell number in a sample of the population, as well as the number of cells of the individual subset(s) of interest within the same sample or sample of equivalent volume and cell concentration.

If the sample contains a defined number of subset cell types, reagents may be prepared containing specific binding substances directed to characteristic antigens of each subset type of interest in order to determine the total cells of interest in the population. One reagent carrier can be prepared which incorporates more than one specific binding substance, permitting the reagent to specifically react with more than one subset cell type. For example, a number of antibodies which interact selectively with the characteristic antigens of a defined number of subset cell types may be incorporated into the reagent preparation. The final reagent is selectively reactive with and capable of forming complexes with all subset

cell types of interest within the population, so that the total number of cells of interest in the population may be determined. A second reagent is prepared which selectively interacts with only one subset cell type of interest.

The total cell number determination may be carried out using the multi-determinant first reagent in a sample of the cell population. Determination of an individual subset cell type of interest within the population may be carried out in a second sample of the population, having equivalent volume and cell concentration. Determination of the reporter substance from each reaction provides an indication of the proportional occurrence of the individual subset cell type(s) within the population. The proportional occurrence determination can also be carried out in a single sample of the population, by incubating the multi-determinant reagent and the subset cell type-specific reagent with the same sample, provided that the type-specific reagent is selected such that it does not appreciably interfere with the action of the multi-determinant reagent and that distinct and separable reporter substances are associated with the type-specific and multi-determinant reagents.

The following examples are provided to describe the invention in further detail. These examples are intended to illustrate certain aspects of the invention and should in no way be construed as limiting the invention. All solvent proportions are given by volume and all temperature in °C, unless otherwise indicated.

Example I: Preparation of liposomes with stably associated reporter substance

The following were mixed in a 1.5 ml Eppendorf® conical centrifuge tube:

a. 10 μmoles dipalmitoyl phosphatidylcholine (DPPC; Sigma, St. Louis, MO) dissolved in 9:1 chloroform:methanol at 50 mg/ml

b. 5 μmoles cholesterol (Sigma, St. Louis, MO) dissolved in 9:1 choroform:methanol at 50 mg/ml

c. 0.15 μmoles dipalmitoyl phosphatidylethanolamine 3-(2-pyridylthio)-propionate (DPPE-DTP), prepared as described by A. Truneh et al., J.Immunol. Method 100: 59-71 (1987), dissolved in 9:1 choroform:methanol at 4 mM

d. 1 ml $DiOC_{18}[3]$ (Molecular Probes, Eugene, OR), as the reporter substance, dissolved in methanol at 0.005 mg/ml

Solvents were evaporated under a stream of dry nitrogen. Evaporation may be carried out at ambient temperature, or with the centrifuge tube immersed in a water bath at about 37° to speed removal of solvent. Solvent removal was completed by lyophilization for 30 minutes. To the dried lipids were added 1 ml of L buffer (145 mM NaCl containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered at pH 8.0). The buffered lipids were then warmed to 50°, and suspended by vigorous vortex mixing. The resulting suspension was sonicated for 15 minutes to form small unilameller liposomes using a cup horn sonicator (Branson Sonic Power Co., Danbury, CN; settings: Power=50, Output=4) with the tube suspended in a 50° bath.

The liposome preparation thus produced was centrifuged for 5 minutes at 12,000 x g, 4° to pellet large liposomes and any $DiOC_{18}[3]$ aggregates.

Small liposomes with stably associated $DiOC_{18}[3]$ in the resulting supernatant were separated from unincorporated $DiOC_{18}[3]$ by chromatography on a PD10 column (prepacked Sephadex G25; Pharmacia, Piscataway NJ) which was previously equilibrated with L buffer at pH 7.5-8.0. The liposome peak elutes before any free reporter substance and is easily visualized due to incorporated reporter substance. With the ratios of reporter substance and lipids here described, no free reporter substance band was observed, suggesting that essentially all $DiOC_{18}[3]$ became associated with the lipid bilayer.

$DiOC_{18}[3]$ appeared to be stably associated with the liposomes, as determined by inability to detect any free fluorochrome peak when a 12 day old preparation was rechromatographed on a PD10 column. Fluorescence intensity of fractions diluted in pH 7.4 L buffer was determined using an SPF 500C spectrofluorometer (SLM-Aminco, Champaign, IL; excitation @ 480nm, emission @ 500nm, high voltage 685, gain 10). All detectable fluorescence was associated with the liposome fractions.

Example II: Preparation of Antibody-Liposome-Reporter Conjugate

Rat anti-mouse Lyt-1 (ATCC #TIB104) was purified from hybridoma culture supernatants. Immunoglobulins were precipitated with 50% v/v saturated $(NH_4)_2SO_4$, dialyzed against 0.02 M K_2HPO_4(pH 8), and chromatographed on an Affigel Blue column (Bio-Rad #153-7307, Richmond, CA). After elution with 0.02 M K_2HPO_4(pH 8) containing 1.4M NaCl, antibody was dialyzed extensively in pH 7.5 L buffer to remove

free amines and suspended at 1 mg/ml protein in the same buffer.

To 1 ml antibody solution in a 12x75 mm glass tube was added, with rapid mixing by brief vortexing, sufficient 20 mM N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP; Sigma, St. Louis, MO) freshly dissolved in dry methanol to obtain a 5:1 mole ratio SPDP:antibody.

The antibody-SPDP mixture was incubated for 30 minutes at room temperature (about 20°). The reaction mixture was adjusted to pH about 4.5 by addition of 80 μl 100mM sodium acetate adjusted to pH 3.3.

To the SPDP-modified antibody was added 50 ´ 1 0.5M dithiothreitol (DTT; Sigma, St. Louis, MO), followed by incubation for 15-20 minutes at room temperature. The thus activated SPDP-antibody was separated from unreacted SPDP by passage through a PD10 column preequilibrated with pH 8 L buffer. Fractions containing the protein peak, as indicated by absorbance at 280nm, were pooled.

SPDP-modified antibody was mixed with the liposome preparation produced in Example I and incubated overnight (about 18-20 hours) in the dark at room temperature to allow covalent coupling to take place.

The antibody-liposome-reporter conjugates (which were visibly colored) were separated from uncoupled antibody by chromatography on a Sepharose 4B column (30 x 1 cm) preequilibrated with pH 7.5 L buffer. For storage, the antibody-liposome-reporter conjugates may be filtered through 0.22 μm sterile Millipore filters presaturated with pH 7.4 L buffer containing 0.1% bovine serum albumin (BSA; Fraction V, Sigma, St. Louis, MO) and maintained at 4°.

Example III: Single Color Cell Labeling and Enumeration With Antibody-Liposome-Reporter Conjugate

2×10^6-1×10^7 Balb/c or CBA2 splenocytes were resuspended in 100µl of the antibody-liposome-reporter conjugate, prepared as described in Example II, diluted 1:5 in phosphate buffered physiological saline containing 0.1% BSA and 0.01% NaN_3 (PBA) and incubated on ice for 60-90 minutes.

The cells were washed 3 times with 1 ml PBA by centrifugation and resuspension. The cells were then fixed by resuspension at 1×10^7/ml in 2% paraformaldehyde.

Cell analysis may be carried out by fluorescence microscopy, flow cytometry, or fluorochrome extraction. The following results were obtained using Balb/c splenocytes stained as described above.

(i) For analysis by flow cytometry using excitation @488 nm and detection @ 530±30nm, the result was 55 ± 2.5% positive (Balb/c)

(ii) For analysis by extraction:

a. cells were pelleted by centrifugation for 5 min. @ 250xg and supernatant was completely removed;

b. cell pellet was resuspended in 2ml 100% ethanol, capped, and vortexed vigorously;

c. cell debris was pelleted by centrifugation for 5 min. @ 250xg; and

d. fluorescence intensity was determined as a function of cell number extracted using an SPF500C spectrofluorometer (excitation @ 480nm, emission @ 500nm, high voltage 685, gain 100). The resulting data are shown in Fig. 1.

Other extractants may be used, if desired, instead

of ethanol, which has a relatively high volatility and is miscible with aqueous solutions, although it is commonly available and presents few disposal problems. An extractant which is non-miscible with aqueous solutions (e.g. butanol) would be expected to give results that are less sensitive to variation in the degree of contamination by aqueous supernatant. A more lipid-like extractant would be expected to give higher fluorescence readings than ethanol, based on the observation that relative fluorescence of the liposome preparation of Example I, above, measured with fluorochrome embedded in intact liposomes was 3.5 times greated than that measured after extraction into ethanol.

Example IV: Multiple Color Cell Enumeration Using Antibody-Liposome-Reporter Reagents

Another antibody-liposome-reporter was prepared as generally described in Example I, by substituting 1 ml 2×10^{-4}M N-[3-sulfopropyl]-4-[p-didecylaminostyryl] pyridinium, inner salt (S467 Molecular Probes, Eugene, OR) for $DiOC_{18}[3]$, and anti-H2K^k antibody, as described by Truneh et al., J. Immunol. Meth., 100:59-71 (1987), for anti-Lyt-1 antibody.

The following cell suspensions were prepared:

a. 100% Balb/c splenocytes (H-2K^d)
b. 100% B10.A splenocytes (H-2K^k)
c. 75% Balb/c + 25% B10.A splenocytes
d. 50% Balb/c + 50% B10.A splenocytes
e. 25% Balb/c + 75% B10.A splenocytes

3×10^7 cells of each of the above cell preparations were resuspended in each of the following reagents, stained, washed and fixed as described in

Example III above (final concentration in fixative $2x10^7$/ml):

Reagent 1: 50 μl Lyt1-liposomes-DiOC_{18}[3]

Reagent 2: 50 μl PBS + 5 l H2k-liposomes-S467

Reagent 3: 50 μl Lyt-1-liposomes-DiOC_{18}[3] + 5 μl $H2K^k$ liposomes-S467

Analysis by flow cytometry: aliquots of $3x10^6$ cells stained with Reagent 3; excitation @ 488nm; emission @ 530±30nm for DiOC_{18}[3], ≥570nm for S467; gave the following results:

sample a: 33% Lyt-1+, 0% $H2K^k$+
sample b: 25% Lyt-1+, 100% $H2K^k$+
sample c: 30% Lyt-1+, 30% $H2K^k$+
sample d: 32% Lyt-1+, 52% $H2K^k$+
sample e: 27% Lyt-1+, 72% $H2K^k$+

Analysis by fluorochrome extraction: aliquots of 10^7 cells stained with Reagent 1 or Reagent 2 were extracted into ethanol as described in Example III, and measured with excitation @ 488nm; emission @ 500nm for DiOC_{18}[3] and 615nm for S467; gain 100 and high voltage 1000 using SPF 500C spectrofluorometer. Mean intensity at 500 nm for samples a-e stained with Reagent 1, was 0.293±0.04. The results at 615nm for samples (a)-(e), stained with Reagent 2, are shown in Fig. 2.

While certain aspects of the present invention have been described and exemplified above as preferred embodiments, various other embodiments will be apparent to those skilled in the art from the foregoing disclosure. Thus, in addition to identification of specific immune cell subtypes, the present invention may be used for subtyping in any other system where a panel of monoclonal antibodies is available, e.g.,

identification of bacterial, fungal, viral and parasitic antigens.

Extraction as a means of quantification of reactivity to a given antigen is also possible using antibody-liposome-reporter conjugates in place of radiolabeled antibodies which are currently used. For example, Radio Allergen Sensitivity Testing (RAST) is widely used to determine the allergens to which an individual is sensitized. A putative allergen is bound to a bead, plate, or other solid substrate and then incubated with patient serum. All antibodies in the patient's serum recognizing that allergen become bound. After washing, further incubation with a radiolabeled anti-IgE allows selective detection of the presence in patient serum of IgE antibodies (i.e., those involved in the allergic response) reactive with the particular allergen. Replacement of radiolabeled anti-IgE with an anti-IgE liposome-reporter conjugate would meet the concern of many clinicians about the use of radioactive reagents. Automation using a robotic system, for example, Beckman Biomek, would be useful for laboratories carrying out large number of such tests and/or determining titers of IgE (level of patient sensitivity), which require multiple dilutions of serum for each allergen.

Clearly the invention described herein may be applied to the determination or enumeration of any antigen which is available in purified form and capable of coupling (either covalently or by adsorption) to a suitable solid support.

The present invention may also be utilized, if desired, in applications requiring high sensitivity, i.e., where detection of very low levels of antigen is

desired. In addition, signal to noise ratios in extracted samples could be improved significantly by using phosphorescent dyes instead of fluorescent ones in the practice of this invention, thereby increasing the counting times. A greater flexibility in selection of spectral properties is possible using the reagents of the invention as compared with using rare earth chelates for delayed fluorescence.

The present invention is, therefore, not limited to the embodiments specifically described and exemplified, but is capable of variation and modification without departure from the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A reagent for determining or quantitatively enumerating analyte having at least one characteristic determinant, said reagent comprising at least one specific binding substance capable of selective interaction with said characteristic determinant, said specific binding substance being coupled to a reporter moiety comprising a detectable reporter substance and a carrier for said reporter substance, said carrier having a lipid component, said reporter substance being stably associated with the lipid component of said carrier.

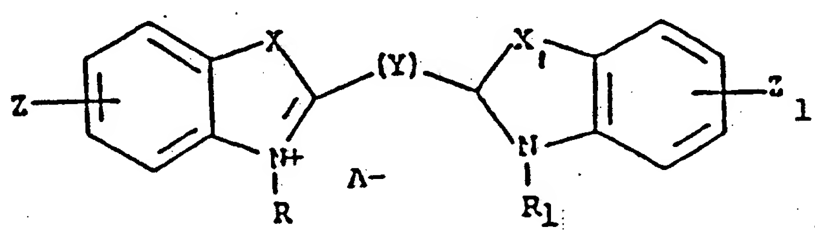
2. A reagent as claimed in claim 1, wherein said carrier is a liposome.

3. A reagent as claimed in claim 2 wherein said reporter substance is a luminescent material.

4. A reagent as claimed in claim 2, wherein said reporter substance is a light absorbing material.

5. A reagent as claimed in claim 2, wherein said reporter substance is a fluorochrome selected from the group of cyanine, acridine, pyridine, anthraquinone, coumarin, quinoline, xanthene, phenoxazine, phenothiazine and hexatriene dyes and derivatives thereof.

6. A reagent as claimed in claim 2, wherein said reporter substance is a compound having the formula:



wherein R and R_1 are the same or different and represent substituents independently selected from the group of hydrogen, alkyl, alkenyl, alkynyl, alkaryl or aralkyl, the hydrocarbon chains of which having from 1 to 30 carbon atoms, and being linear or branched, said substituents being unsubstituted or substituted with one or more non-polar functional groups, one of R or R_1 having at least 12 linear carbon atoms, and the sum of the linear carbon atoms in R and R_1 being at least 23;

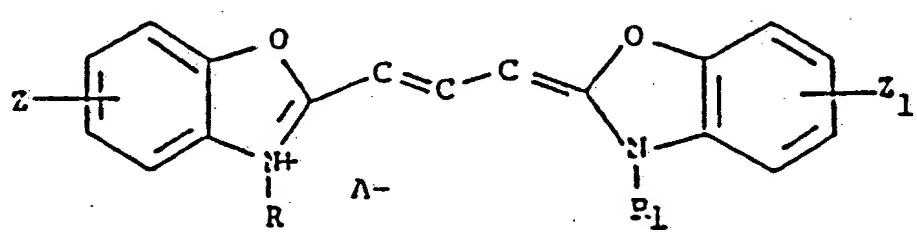
X and X_1 may be the same or different and represent O, S, $C(CH_3)_2$ or Se;

Y represents a linking group selected from -CH=, -CH=CH-CH=, -CH=CH-CH=CH-CH=, or -CH=CH-CH=CH-CH=CH-CH=;

Z, and Z_1 are the same or different and represent substituents selected from the group H, alkyl, OH, NH_2, COOH, $CONH_2$, SO_3H, SO_2NH_2, $NHNH_2$, NCS, NCO, SO_2NH_2, CONH-alkyl, CON-$(alkyl)_2$, NH-acyl, -O-alkyl, NH-alkyl, $N(alkyl)_2$, SH, S-alkyl, NO_2 or halogen, the alkyl groups comprising said Z substituents having from 1 to 3 carbon atoms; and

A represents a biologically compatible anion.

7. A reagent as claimed in claim 2, wherein said reporter substance is a compound having the formula:



wherein R and R_1 are the same or different and

represent alkyl substituents, having from 1 to 30 carbon atoms, and being linear or branched, unsubstituted or substituted with halogen, one of R or R_1 having at least 12 linear carbon atoms and the sum of the linear atoms in R and R_1 being at least 23;

Z and Z_1 are the same of different and represent substituents selected from the group H, or lower alkyl having from 1 to 3 carbon atoms; and

A represents a biologically compatible anion.

8. A reagent as claimed in claim 2, wherein said reporter substance is 3,3' di-n-octadecyloxacarbocyanine perchlorate.

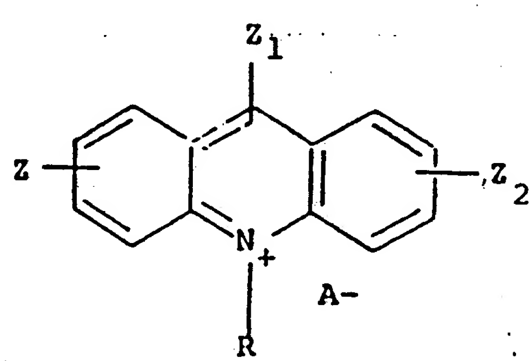
9. A reagent as claimed in claim 2, wherein said reporter substance is 3-n-Pentyl-3'-n-octadecyloxacarbocyanine Iodide.

10. A reagent as claimed in claim 2, wherein said reporter substance is 3-n-Octyl-3'-n-octadecyloxacarbocyanine Iodide.

11. A reagent as claimed in claim 2, wherein said reporter substance is 3-n-Propyl-3'-n-eicosanyloxacarbocyanine Iodide.

12. A reagent as claimed in claim 2, wherein said reporter substance is 3-n-Propyl-3'-n-docosanyloxacarbocyanine Iodide.

13. A reagent as claimed in claim 2, wherein said reporter substance is a compound having the formula:



wherein R represents a substituent selected from the

group of alkyl, alkenyl, alkynyl, alkaryl or aralkyl, the hydrocarbon chain of which is linear or branched, said substituent being unsubstituted or substituted with one or more non-polar functional groups, and having at least 23 linear carbon atoms;

Z, Z_1 and Z_2 are the same or different and represent substituents selected from the group H, alkyl, OH, NH_2, COOH, $CONH_2$, SO_3H, SO_2NH_2, $-NHNH_2$, -NCS, -NCO, CONH-alkyl, CON-$(alkyl)_2$, NH-acyl, -O-alkyl, NH-alkyl, N$(alkyl)_2$, SH, S-alkyl, NO_2, or halogen, the alkyl groups comprising said Z substituents having from 1 to 3 carbon atoms; and

A represents a biologically compatible anion.

14. A reagent as claimed in claim 2, wherein said reporter substance is 3,6-bis(dimethylamino)-10-n-hexacosanyl acridinium iodide.

15. A reagent as claimed in claim 2, wherein said reporter substance is 4-[4-didecylaminostyryl]-N-methylpyridinium iodide.

16. A reagent as claimed in claim 2, wherein said reporter substance is N-[3-Sulfopropyl]-4-[p-didecylaminostyryl] pyridinium, inner salt.

17. A reagent as claimed in claim 2 wherein said reporter substance comprises a chelate-metal complex.

18. A reagent as claimed in claim 17, wherein said chelate-metal complex comprises a metal ion selected from the transition metal series whose atomic number is from 21-29, the lanthanide series whose atomic number is 59-66 and the actinide series whose atomic number is 91, said complex being detectable by nuclear magnetic resonance or luminescence.

19. A reagent as claimed in claim 18, wherein

said chelate-metal complex comprises a paramagnetic metal ion selected from the group of Gd, Cr, Dy, Ni, Cu, Fe and Co.

20. A reagent as claimed in claim 2, wherein said reporter substance comprises a radioisotope.

21. A reagent as claimed in claim 20, wherein the radioisotope constituent of said reagent is selected from the group of radioactive hydrogen, carbon, nitrogen, phosphorus, fluorine, chlorine, iodine, sulphur and selenium.

22. A reagent as claimed in claim 2, wherein additional reporter substance is encapsulated within said liposome.

23. A reagent as claimed in claim 1, wherein said specific binding substance comprises at least one antibody.

24. A reagent as claimed in claim 23, wherein said antibody is a monoclonal antibody.

25. A reagent as claimed in claim 23, wherein said antibody interacts selectively with analyte selected from the group of viral, bacterial, fungal and parasitic antigens.

26. A reagent as claimed in claim 25, wherein said antibody is a monoclonal antibody.

27. A reagent as claimed in claim 23, wherein said specific binding substance comprises at least one antibody fragment.

28. A method for detecting the presence of or quantitatively enumerating analyte having at least one characteristic determinant, in a sample, comprising:

a) providing a reagent comprising at least one specific binding substance capable of selective interaction with said determinant, said

specific binding substance being coupled to a reporter moiety comprising a detectable reporter substance and a carrier for said reporter substance, said carrier having a lipid component, said reporter substance being stably associated with the lipid component of said carrier,

b) contacting said reagent with said sample under conditions causing binding of said specific binding substance and said determinant;

c) separating unbound reagent from said sample;

d) determining the occurrence of said reporter substance in the resulting sample or in said separated unbound reagent by detecting or quantitating said reporter substance to provide an indication of the presence or quantity of said analyte in said sample.

29. A method as claimed in claim 28, wherein said reagent is provided having a fluorescent compound as said reporter substance and a liposome as said carrier.

30. A method as claimed in claim 28, wherein said reagent is provided having additional reporter substance encapsulated within said liposome.

31. A method as claimed in claim 28, wherein said reporter susbstance is extracted from said carrier prior to detection or quantitation thereof.

32. A method for detecting or quantitatively enumerating cell-expressed ligand, comprising:

a) providing at least one reagent comprising specific binding substance capable of selective interaction with said ligand, said binding substance being coupled to a liposome having a reporter substance stably associated with the lipid component of said liposome;

b) contacting said reagent with a sample of said cells under conditions causing binding of said binding substance and said ligand to form complexes;

c) separating unbound reagent from said complexes;

d) detecting or quantitating the occurrence of the reporter substance in the resulting complexes or in the separated unbound reagent to provide an indication of the presence or quantity of immunoreactive material expressed on the surfaces of said cells.

33. A method as claimed in claim 32, wherein said reagent is provided having antibody as the specific binding substance for detecting or quantitatively enumerating cell surface antigen.

34. A method as claimed in claim 32, wherein said reagent is provided having a fluorescent compound as the reporter substance.

35. A method as claimed in claim 32, wherein said reagent is provided having additional reporter substance encapsulated within said liposome.

36. A method as claimed in claim 32, wherein said reporter substance is extracted from said liposome prior to detection or quantitation thereof.

37. A method for detecting or quantitatively enumerating a subset cell type in a cell population, said subset cell type being characterized by the expression of a characteristic antigen, comprising:

a) providing at least one reagent comprising antibody which specifically interacts with said antigen, said antibody being coupled to a liposome

having a reporter substance stably associated with the lipid component of said liposome;

b) contacting said reagent with a sample containing said cell population under conditions causing binding of said antibody and said antigen to form complexes;

c) separating unbound reagent from said complexes;

d) detecting or quantitating the occurence of the reporter substance in the resulting complexes or in the separated unbound reagent to provide an indication of the presence or quantity of said subset cell type within said population.

38. A method as claimed in claim 37, wherein said reagent is provided having a fluorescent compound as the reporter substance.

39. A method as claimed in claim 37, wherein said reagent is provided having additional reporter substance encapsulated within said liposome.

40. A method as claimed in claim 37, wherein said reporter substance is extracted from said liposomes prior to detection or quantitation thereof.

41. A method as claimed in claim 37, wherein said cell population consists of leukocytes, erythrocytes or mixtures thereof.

42. A method as claimed in claim 37, wherein said population consists of hemopoietic cells, and said subset cell type consists of hemopoietic cells of a certain lineage or stage of differentiation.

43. A method as claimed in claim 37, wherein said cell population consists of T cells and said subset cell type consists of T cells bearing certain characteristic antigens indicative of cell function or

stage of differentiation.

44. A method as claimed in claim 37, which comprises determining or quantitatively enumerating multiple subset cell types from said population, each subset cell type expressing a characteristic antigen, by providing a reagent for each subset cell type to be determined or enumerated, each reagent comprising antibodies reactive to a characteristic antigen of the subset cell type for which each said reagent is provided, said antibodies being coupled to liposomes, the reagent for each subset cell type having a reporter substance stably associated with said liposomes and distinguishable from the reporter substance of the other reagents.

45. A method as claimed in claim 44, wherein the reporter substance of each reagent is a fluorescent compound having distinct spectral properties.

46. A method as claimed in claim 44, wherein at least one of the reagents is provided having additional reporter substance encapsulated within said liposome.

47. A method of preparing a reagent comprising specific binding substance for use in detecting the presence of or quantitatively enumerating analyte having at least one characteristic determinant that interacts selectively with said binding substance comprising:

a) preparing liposomes incorporating a lipophilic reporter substance in stable association with the lipid component of said liposomes; and

b) contacting said liposomes incorporating said reporter substance with said binding substance, under conditions causing said liposomes to couple to said binding substance thereby forming said reagent.

48. A method as claimed in claim 47, wherein said coupling is effected by covalent linkage selected from a disulfide, ester, amide, triazine, acetal, imine, hydrazone, semicarbazone, urea, thiourea, ether, thioether, linkage or a reduced imine linkage.

49. A method as claimed in claim 47, wherein sulfhydryl groups are incorporated in said liposomes and said binding substance, said sulfhydryl groups being reactive to form a disulfide bond between said liposomes and said binding substance.

50. A method as claimed in claim 47, wherein said specific binding substance is antibody Fab domain, which is stably associated with said liposomes through a lipophilic compound coupled to the Fc domain of the same antibody.

51. A method for determining the proportion of individual subset cell types relative to a cell population, said cell population consisting of a defined number of subset cell types of interest, and wherein each said subset cell type has at least one characteristic ligand, comprising:

(a) providing a first reagent comprising one or more specific binding substances that interact selectively with at least one characteristic ligand of each subset cell type of interest within said cell population, said binding substance being coupled to a liposome having a reporter substance stably associated with the lipid component of said liposome;

(b) contacting said first reagent with a first sample containing said cell population under conditions causing binding of the binding substance of said first reagent and said characteristic ligands, thereby forming first complexes between first reagent and all subset cell types of interest;

(c) separating unbound first reagent from

said first complexes;

(d) determining the occurence of the reporter substance in the resulting first complexes or in the separated unbound first reagent by separating the lipid fraction containing said reporter substance therefrom and quantitating said reporter substance to provide an indication of the quantity of cells in the population;

(e) providing an additional reagent for each individual subset cell type to be determined, comprising a specific binding substance that selectively interacts with a characteristic ligand of the individual subset cell type being determined, said binding substance of each additional reagent being coupled to a liposome having a reporter substance stably associated with the lipid component of said liposome;

(f) contacting the additional reagent with a sample of said cell population of equivalent volume and cell concentration to said first sample, under conditions causing binding of the binding substance of said additional reagent and said characteristic ligand of the individual subset cell type in the sample with which said additional reagent is contacted, thereby forming second complexes in said sample;

(g) separating unbound additional reagent from the second complexes in said sample;

(h) determining the occurrence of the reporter substance in said complexes in said sample by separating the reporter substance from the complexes and quantitating said reporter substance to provide an indication of the quantity of cells of said individual subset cell type within said sample; and

(i) determining the proportional

occurrence of said individual subset cell type relative to said population on the basis of the quantity of reporter substance determined using said first reagent and the quantity of reporter substance determined using the additional reagent.

52. A method for determining the proportion of individual subset cell types in a sample containing a cell population, said cell population consisting of a defined number of subset cell types of interest, and wherein each said subset cell type has at least one characteristic antigen, comprising:

(a) contacting the sample with a first reagent comprising at least one specific binding substance that selectively interacts with at least one characteristic antigen of each subset cell type of interest within said cell population, said binding substance being coupled to a liposome having a first reporter substance stably associated with the lipid component thereof, under conditions causing binding of the binding substance of said first reagent and said characteristic antigen to form complexes between first reagent and all subset cell types of interest;

(b) contacting the sample with an additional reagent for each individual subset cell type to be determined comprising specific binding substance that selectively interacts with a characteristic antigen of the individual subset cell type being determined, said binding substance being selected such that its interaction with the individual subset cell type antigen to which it binds does not appreciably interfere with complex formation between said binding substance of said first reagent and the antigen with which it selectively interacts, said binding substance of each additional reagent being coupled to a liposome having a reporter substance, which is distinguishable from said first

reporter substance and from the reporter substance of any other additional reagent, stably associated with the lipid component of said liposome, said contacting of sample and additional reagent occurring under conditions causing binding of the binding substance of each additional reagent and said characteristic antigen of the individual subset cell type in said sample with which the binding substance of each additional reagent specifically interacts, to form complexes between additional reagent and the individual subset cell type of interest;

(c) separating unbound reagent from said complexes;

(d) determining the relative quantities in the resulting complexes or in the separated unbound reagent of said reporter substance from said first reagent and said reporter substance from each said additional reagent to indicate the proportional occurrence of each individual subset cell type in the cell population.

53. A method as claimed in claim 52, wherein said first reagent and said additional reagent are provided having fluorescent compounds as the reporter substances.

54. A method as claimed in claim 52, wherein at least one reagent is provided having additional reporter substance encapsulated within said liposome.

55. A method as claimed in claim 52, wherein the sample is contacted with a further reagent comprising at least one specific binding substance capable of selective interaction with antigen of another individual subset cell type, said specific binding substance being coupled to a reporter moiety comprising a detectable hydrophilic reporter substance encapsulated within a liposome wherein said hydrophilic reporter is distinguishable from other reporters on the basis of solubility in aqueous solutions.

56. A method as claimed in claim 52, wherein said reporter substance is extracted from said liposomes

prior to quantitation thereof.

57. A method as claimed in claim 52, wherein the reporter substance of said first reagent and the reporter substance of each said additional reagent are luminescent compounds having different excited state lifetimes.

58. A method as claimed in claim 52, wherein the reporter substance of said first reagent and the reporter substance of each said additional reagent are fluorescent compounds having different spectral properties.

59. A method for determining or quantitatively enumerating analyte having at least one characteristic determinant which is capable of binding selectively to a complementary binding substance, in a sample suspected of containing said analyte, comprising:

(a) providing a reagent comprising said complementary binding substance coupled to a liposome having a fluorescent substance stably associated with the lipid component of said liposome;

(b) contacting said reagent with said sample under conditions causing binding of the analyte to said complementary binding substance of said reagent;

(c) separating unbound reagent from the sample; and

(d) detecting or quantitating fluorescence of said fluorescent substance, to provide an indication of the presence or quantity of analyte in said sample.

60. A method as claimed in claim 59, wherein said fluorescent substance is extracted from said liposome prior to detection or quantitation thereof.

61. A method as claimed in claim 59, wherein

said analyte is antigen and said complementary binding substance is antibody.

62. The method as claimed in claim 61, wherein said antibody consists of monoclonal antibody.

63. The method as claimed in claim 61, wherein said antibody comprises at least one antibody fragment.

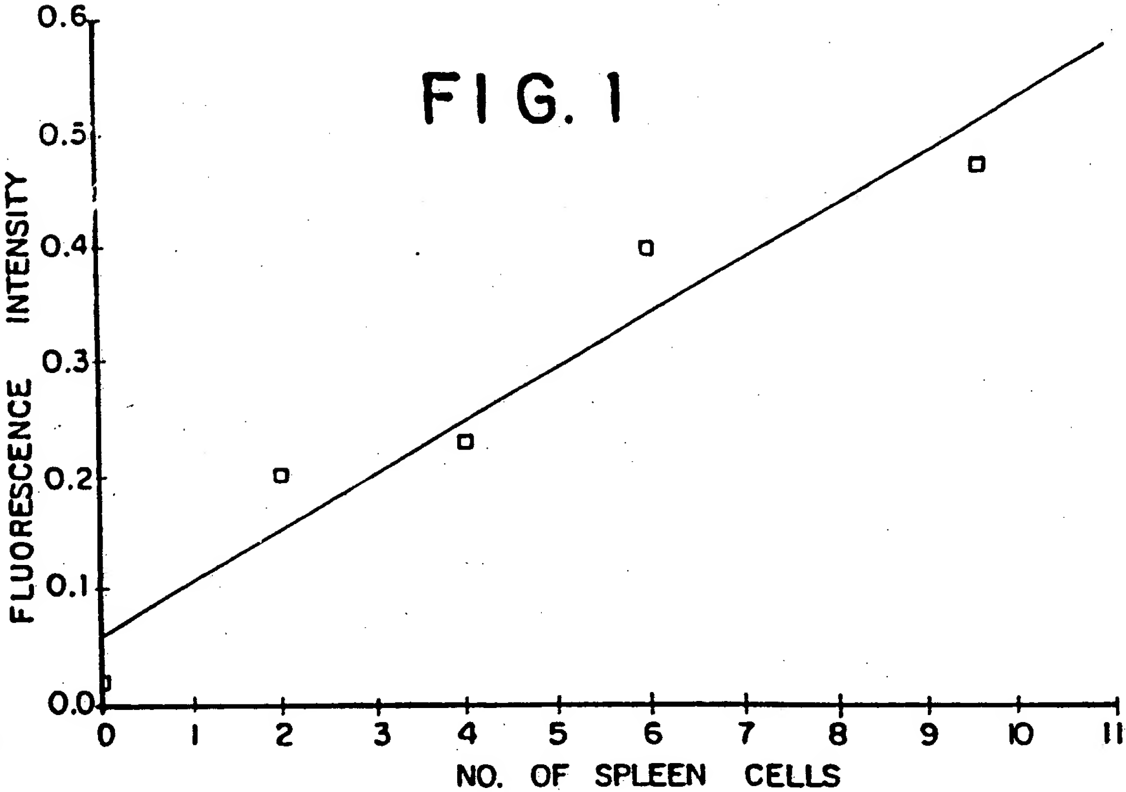

FLUORESCENCE INTENSITY
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
1
2
3
4
5
6
7
8
9
10
11
NO. OF SPLEEN CELLS

FIG. 1

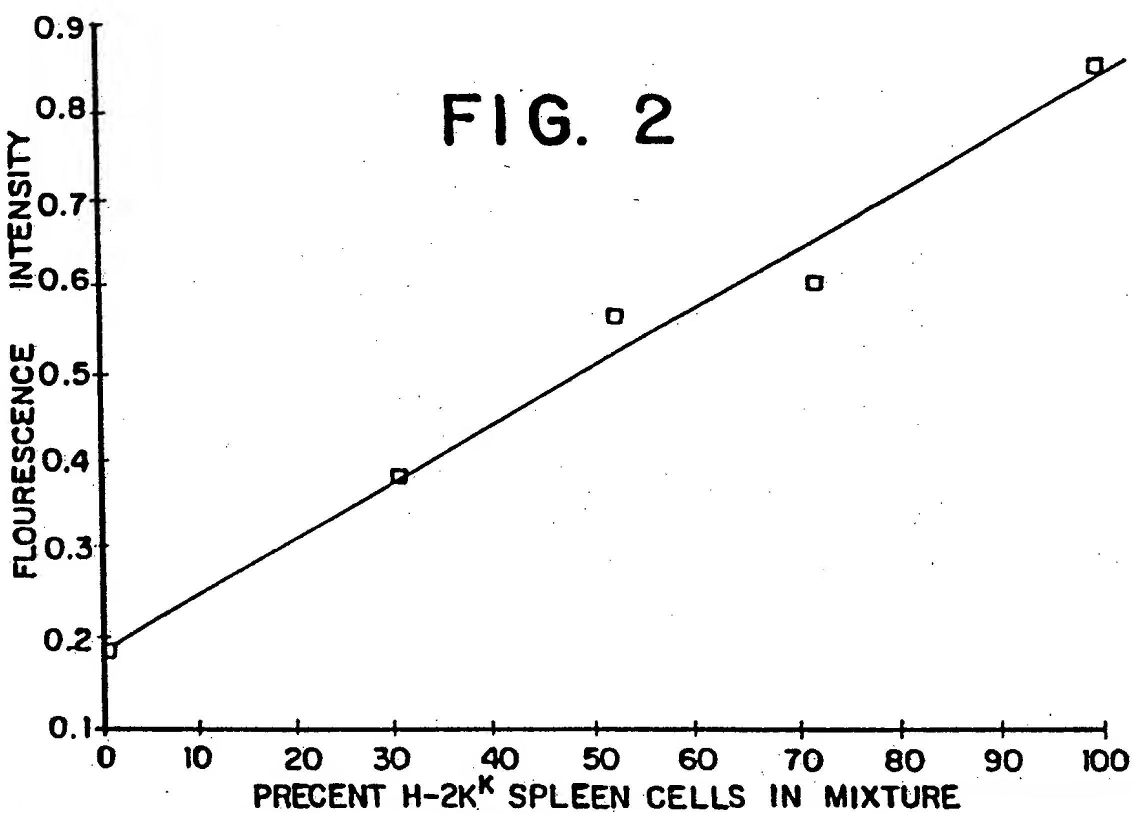

FLOURESCENCE INTENSITY
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
10
20
30
40
50
60
70
80
90
100
PRECENT H-2K^K SPLEEN CELLS IN MIXTURE

FIG. 2

I..ERNATIONAL SEARCH REPOR.

International Application No. PCT/US89/03727

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 33/535, G01N 33/543, A01N 1/02
US Cl.: 435/7, 436/518, 427/2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5,7,810 264/4,4.1,4.3 436/518,520,521,522,528,544,547,800,805,829,512 427/2,3

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

COMPUTER SEARCH: MEDLINE, BIOSIS PREVIEWS, WORLD PATENTS INDEX

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,698,263 (Wagner) 06 October 1987, see column 1, lines 29-44, column 3, lines 27-32 and 40-53, column 7, lines 38-54 and column 9, lines 18-28.	1-63
Y	US, A, 4,636,479 (Martin) 13 January 1987, See paragraph bridging columns 6 and 7, column 9, lines 19-27 and column 8, lines 48-53.	1-63
Y	US, A, 4,560,665 (Nakae) 24 December 1985, see column 1, lines 43-53 and the paragraph bridging columns 2 and 3.	5,8-12
Y	US, A, 4,707,453 (Wagner) 17 November 1987, see column 4, lines 43-column 5, line 13 and column 7, lines 19-25.	1-63

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

24 November 1989

Date of Mailing of this International Search Report

08 DEC 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

Esther M. Kepplinger

ESTHER M. KEPPLINGER

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,342,739 (Kakimi) 03 August 1982, see column 2, lines 31-34 and column 6, line 37-column 7, line 54.	1-63
Y	Journal of Immunological Methods, Volume 100, No. 1-2, 59-71, issued 1987, A. Truneh et al, "Antibody-Bearing Lipsomes as Multicolor Immunofluorescence Markers for Flow Cytometry and imaging", see page 61, column 1, page 67, column 2, page 68, column 1 and page 69, column 2.	1-63

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3.☐ Claim numbers ____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.